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Skeletal aging and the adipocyte program

New insights from an "old" molecule

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Aging is associated with profound changes in bone mass and body composition. Emerging evidence supports the hypothesis that alterations in mesenchymal stromal cell fate are a critical etiologic factor. In addition, timekeeping at the cellular level is affected as aging progresses, particularly in the adipocyte. In this Extra View we discuss the interactive role of three molecules, PPARγ, nocturnin and IGF-I, in regulating stem cell fate in the marrow and the potential implications of this network for understanding cellular aging.

Key words: aging, bone, PPARγ, nocturnin, IGF-I

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Introduction

Advanced aging in mammals is associated with profound changes in both body composition and bone mass.[1-3] In the former, fat redistribution is noticeable such that muscle, liver and bone show evidence of fatty deposition and subcutaneous depots frequently atrophy as the efficiency of adipose tissue activity in those sites declines. These changes in whole body adiposity may affect the balance between energy storage and energy expenditure which in part is regulated through the adipokine, leptin, a major stimulator of sympathetic activation.[4] Coincident with site-specific changes in adiposity, age-related bone loss accelerates as trabeculae degenerate and the endosteal envelope thins.[5] This results in bone fragility and ultimately osteoporotic fractures. Not surprisingly, the bone marrow microenvironment mirrors these systemic changes as hematopoietic stem cell numbers decline, adipocytes become more prevalent and osteoblastic activity slows. Importantly, the cellular origin of this shift can be recapitulated in vitro by activation of specific transcription factors in mesenchymal stromal cells (MSCs).[6,7] Although lineage allocation into adipocytes or osteoblasts is generally considered as mutually exclusive, little is known about the function of marrow adiposity and its relationship to other adipose depots or the skeleton, particularly with aging.[8-10] One consistent finding is that PPARγ2, a critical adipocytic transcription factor, is upregulated several folds in bone marrow from old vs. adult animals (**Fig. 1**). This correlates closely with the number of marrow adipocytes and inversely with bone mass (**Fig. 2**). These findings are also observed in diabetes mellitus, a disease that often manifests itself in many systems as accelerated aging.[11]

The replacement of marrow stromal elements with fat cells was long considered a passive process with no functional implications. However, this view has undergone considerable revision in light of new insights about the PPARγ regulatory program and use of new mouse models. Recently our laboratories defined an important link between PPARγ and nocturnin (Noc), a circadian regulated gene that evolved millions of years ago from the yeast family of transcription factors regulating cellular responses to external cues.[12-14] The role of this network in the bone micro environment during aging and its relationship to IGF-I informs us about both physiologic and pathologic processes that become operational as the mammalian organism ages.

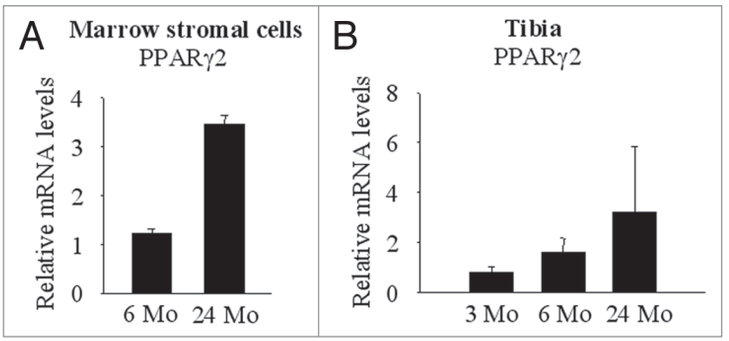

Figure 1. Aging-related increase in PPARγ2 expression in bone marrow stromal cells. (A) Bone marrow stromal cells were collected from adult (6 months old) and old (24 months old) mice and expanded. PPARγ2 expression was measured by real-time PCR. PPARγ2 expression was enhanced in cells from old mice compared to those from adult mice. (B) RNA was extracted from tibia of young (3 mo), adult (6 mo) and old (24 mo) mice. PPARγ2 expression was measured by real-time PCR.

Age-Related Micro-Structural Change in the Bone Marrow

Age-related bone loss occurs universally in all mammals and, unlike postmenopausal bone loss, affects individuals regardless of sex steroid status. Maintenance of bone homeostasis throughout life relies on bone remodeling, which continually replaces old and damaged bone with new bone in order to maintain bone strength and elasticity.[1] Two types of cells are involved in bone remodeling: osteoclasts, which originate from hematopoietic cells, are responsible for bone resorption and osteoblasts, which originate from mesenchymal cells, are responsible for bone formation. The characteristic feature of age-related bone loss is the uncoupling of formation from resorption leading to a net loss of bone mass. The etiology of this uncoupling is multifactorial and includes changes in endogenous gonadal steroids, increased reactive oxygen species and a global decline in local growth factors that promote osteoblastic differentiation.[5,15,16] And, coincident with primary skeletal changes, muscle wasting, which is common during the aging process is another factor affecting bone loss.[17] Interestingly, sarcopenia is associated with muscle atrophy and fatty infiltration.

Bone marrow consists of a number of cell types including hematopoietic stem cells, mature blood cells, adipocytes, endothelial cells, osteoblasts and osteoclasts. The compositional ratio in bone marrow of these cells changes with age. For example, in neonatal mammals, adipocytes are all but absent in the bone marrow and hematopoietic cells primarily occupy the marrow cavity at this stage. During pubertal growth, there is gradual infiltration of marrow from the long bones with adipocytes. However, with advancing age, the number of adipocytes in the bone marrow increases dramatically resulting in the appearance of fatty marrow. In humans, most of the femoral cavity is occupied by fat by the third decade of life, whereas in the vertebrae this does not occur until the 7th or 8th decade. Importantly, these age-related changes in marrow adiposity are associated with bone loss. In insulin-dependent Type I diabetes this inverse correlation between marrow adipocytes and bone mass occurs at a much earlier age.[18] The dynamic changes in the micro-structure of bone marrow with aging are accompanied by impaired osteoblast proliferation resulting in a decreased osteoblast pool and robust infiltration of marrow adipocytes.[10,19] In many cases, osteoclast number is also increased.

Marrow fat has long been considered inert and the default pathway for the cell fate determination of MSCs; however, accumulating evidence demonstrate the emerging role of marrow adipocytes as more than passive occupants of the marrow.[3] Indeed, the Daley laboratory has demonstrated in vivo that marrow adiposity in at least two skeletal sites of the mouse (i.e., tail and vertebrae) serves to inhibit hematopoiesis.[20] Since the phenotype of marrow adipocytes is similar to that of adipocytes present in white and brown fat, the context specific nature and unique location of these cells indicate these cells are likely to have a more specialized function.[21] For example, the expression of pro-inflammatory cytokines increases with aging leading to the tenet that this type of adipose tissue may have a lipotoxic effect on bone.[22] Likewise, the adipokines leptin and adiponectin may also modulate osteoblast differentiation and function in a paracrine manner.[23-29] Thus, marrow adipocytes could be metabolically active and may function as a negative regulator for hematopoiesis and osteoblastogenesis through secretory factors. On the other hand, there are mouse models in which marrow fat is increased and trabecular bone mass is high, or where marrow adiposity is transient, implying there is a dynamic nature to the adipocyte program.[29-31]

PPARγ as a Factor Regulating MSCs Lineage Commitment

One possible mechanism underlying the age-associated micro-structural change in the bone marrow resides in the shift of cell fate determination of MSCs toward adipogenesis.[8-10,33] Osteoblasts and adipocytes share a common precursor cell, but the lineage commitment of MSCs toward adipogenesis and osteoblastogenesis is often mutually exclusive. The determination of MSCs cell fate involves a number of transcription factors, and PPARγ is one such factor regulating this process. PPARγ is a member of the PPAR family of transcriptional factors and nuclear receptors which possess a critical role in adipogenesis and osteogenesis as evidenced by the fact that haploinsufficiency or hypomorphic mutation of PPARγ result in the high-bone mass phenotype and reduced marrow adiposity.[34,35] In addition, as shown in a number of in vitro models of MSC differentiation, as well as in primary bone marrow cells, the activation of PPARγ2 with either natural (fatty acids and eicosanoids) or artificial (TZD) ligands directs MSC differentiation toward the adipocyte lineage at the expense of osteoblast formation.[3,6,32,36] Moreover, activation of PPARγ2 in cells of the osteoblast lineage converts them to terminally differentiated adipocytes and irreversibly

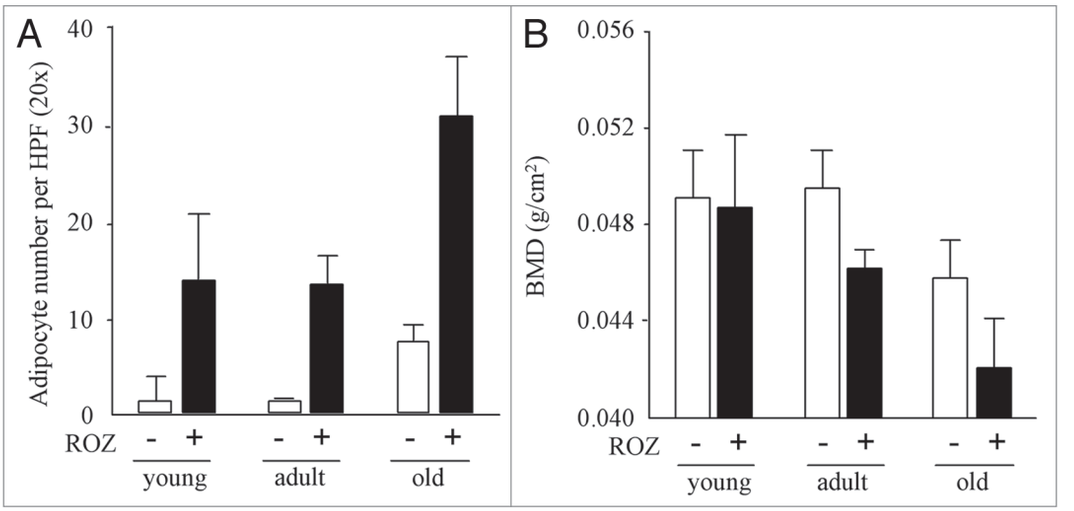

Figure 2. Aging- and rosiglitazone-dependent increase of marrow adiposity and decrease in bone mineral density (BMD). (A) Adipocyte number in the bone marrow was counted in young (3 months), adult (6 months) and old (24 months) mice. Marrow adipocyte number was increased with age, which was further enhanced by the treatment with rosiglitazone (R). (B) Whole body areal bone mineral density (BMD) was measured in young (3 months), adult (6 months) and old (24 months) mice.

suppresses their phenotype, including suppression of osteoblast-specific signaling pathways such as the Wnt, TGFβ/BMP and IGF-1 pathways and transcriptional regulators such as Dlx5, Msx2, Runx2 and Osterix.[6,10,37] Importantly, expression of PPARγ2 increases with age in the bone marrow of mice and this is associated with increased marrow adiposity and bone loss (**Figs. 1 and 2**). Thus, enhanced PPARγ2 expression in the bone marrow milieu plays an important role in the pathogenesis of age-related bone loss through shifting the cell fate of MSCs toward adipogenesis and away from osteogenesis. In addition to the alteration of cell fate determination, phenotypic changes of MSCs during aging process could also be responsible for age-related bone loss. For example, impairment of cell proliferation and differentiation, as well as chromosomal instabilities of MSCs have been implicated in long-term cell culture models.[38]

Activation of bone resorption also plays an important part in age-related bone loss. Since PPARγ has been implicated in the activation of osteoclastogenesis, increases in PPARγ2 expression in the bone marrow could be responsible for the increase in bone resorption with advanced aging.[39-42] In addition, production of macrophage colony-stimulating factor (M-CSF) and RANKL, two pro-osteoclastic cytokines, has been shown to be increased with aging, adding another possible mechanism for increased bone resorption.[43]

Nocturnin as a Modulator for PPARγ Rhythmic Expression

It is well established that there is a strong connection between circadian networks and metabolic outputs; however, the precise mechanisms whereby the circadian system affects metabolic status remains largely unknown.[44] Given the fact that nuclear receptors play critical roles in a wide range of metabolism and most of the nuclear receptors possess a circadian expression profile, it is conceivable that nuclear receptors have an important role in circadian-regulated metabolic responses. In fact, PPARγ is one such factor, which has a profound effect on lipid and glucose metabolisms and exhibits a circadian expression pattern that is amplified by a high-fat diet.[44-46]

Green et al. identified Nocturnin (Noc) as a circadian-regulated gene in Xenopus retina.[47] Noc expression shows peak expression at around the light offset in most tissues including liver, spleen, kidney and skeleton.[48] Noc is a member of a family of proteins that includes transcription factors, deadenylases and phosphatases[49-52] and functions as a deadenylase which degrades mRNA from polyadenylation site of 3'UTR,[50] thus serving as a post-transcriptional mechanism to regulate gene expression. The detailed analyses of metabolic phenotype and body composition of Noc$^{-/-}$ mice revealed that these mice were protected from high-fat diet-induced obesity and fatty liver changes; interestingly, PPARγ circadian expression profile in the liver disappeared in Noc$^{-/-}$ mice on the high-fat diet, implying that PPARγ function is impaired in Noc$^{-/-}$ mice.[14] Consistent with this idea, Noc$^{-/-}$ mice showed high bone mass phenotype and decreased marrow adiposity (**Fig. 3**).[13] These lines of evidence indicate that a circadian-regulated gene, Noc, modulates metabolic outputs and body composition through PPARγ and its circadian expression.

The Role of Nocturnin in the Regulation of MSC Cell Fate

To better understand the role of Noc on PPARγ activity, we analyzed the temporal expression profiles of Noc during adipogenesis and osteogenesis. Noc expression is increased during adipogenesis of 3T3-L1 cells, while suppressed during the osteogenesis of primary calvarial osteoblasts.[13] These data led us to speculate that Noc is a positive regulator for adipogenesis and a negative regulator for osteogenesis. Indeed, overexpression of Noc in 3T3-L1 cells enhances adipogenesis accompanied

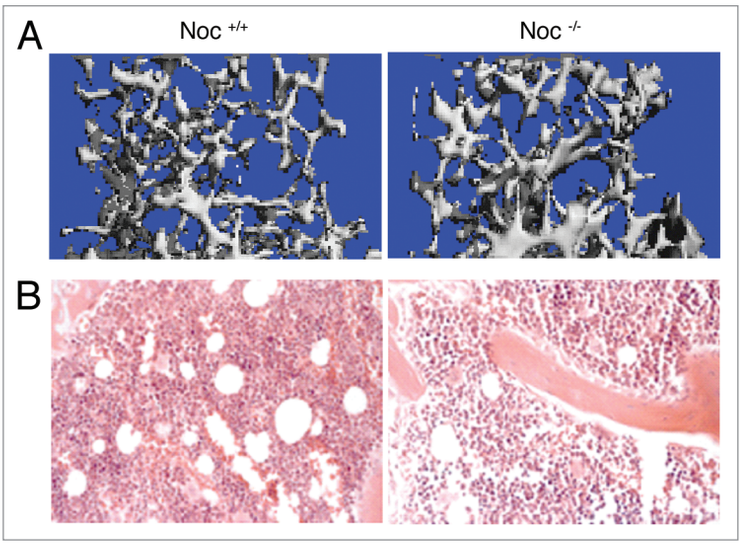

Figure 3. High bone mass and reduced marrow adiposity in Noc$^{-/-}$ mice. (A) Trabecular bone micro-architecture of the distal femur was analyzed by microCT at 16 weeks of animal's age. (B) Marrow adiposity was evaluated in HE-stained femurs of the same animals.

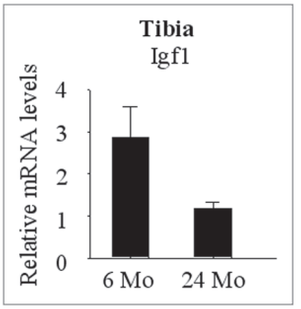

Figure 4. Aging-associated decrease in Igf1 expression in the bone. RNA was collected from tibia of adult (6 months) and old (24 months) mice and Igf1 expression was analyzed by real-time PCR.

by increased PPARγ2 expression. In contrast, overexpression of Noc suppressed osteogenesis of MC3T3-E1 cells.[13] These lines of evidence together with the in vivo phenotype of high-bone mass and decreased marrow adiposity in Noc$^{-/-}$ mice indicate that Noc may regulate specification of MSCs toward the adipogenic lineage. Cell fractionation and immunofluorescence analyses revealed the exclusive expression profile of Noc in the cytoplasm and nuclear membrane, ruling out the possibility that Noc functions as a co-activator for PPARγ.[13] But interestingly, Noc binds to PPARγ and facilitates its entry into the nucleus, which enhances PPARγ transcriptional activity. Interestingly, the effect of Noc on PPARγ activity is independent of its deadenylase activity because the mutant Noc, which lacks deadenylase activity, exhibits the same effect on PPARγ activity.[13] Of note is the fact that Noc is an inducible gene. Noc has been shown to be induced by fetal calf serum, FOXO1 and insulin in NIH3T3 and 3T3-L1 cells,[13,49] suggesting that Noc expression could be affected by energy status and that Noc might be one factor that links external stimuli to metabolic output through PPARγ.

PPARγ Regulation of IGF-I: Implication for the Adipocyte Program and Age-Related Bone Loss

Insulin-like growth factor-I (IGF-I) both locally and in the circulation regulates a number of physiological aspects of skeletal and adipose metabolism.[53,54] For example IGF-I expression is high in proliferating mesenchymal stem cells, but then falls with differentiation into either adipocytes or osteoblasts.[54] Late in bone cell differentiation IGF-I production rises and this is also noted for fully differentiated adipocytes. Circulating IGF-I, which primarily originates from liver synthesis, can also regulate skeletal accrual in an endocrine manner.[55,56] These findings are evidenced mostly by analyses using genetically engineered mouse models, but human clinical studies/observations also indicate the critical role of systemic IGF-I in the regulation of bone mass. For example, a large body of evidence demonstrates the positive correlation between IGF-I serum concentration and bone mass across several key time points of life. Serum IGF-I levels in umbilical cord blood has been related to whole body bone mineral content (BMC) in full-term 119 newborn infants.[57] Furthermore, serum IGF-I levels peak at puberty when skeletal acquisition is maximized and then declines with age as bone mass decreases. Cohort studies have also revealed a positive association of IGF-I serum concentration and bone mass in post-menopausal women.[58,59]

Locally produced IGF-I in the skeletal microenvironment plays a pivotal role in skeletal mass.[60,61] For example, osteoblastic overexpression of *Igf1* results in decreased bone mass without affecting serum IGF-I concentrations.[60,61] Importantly, as observed with circulating IGF-I, there is an age-related decline in skeletal IGF-I concentrations associated with bone loss (**Fig. 4**). These changes in the bone marrow are also accompanied by the infiltration of marrow adipocytes and increased PPARγ2 expression in the bone marrow (**Figs. 1 and 2**). Given the anti-osteogenic capacity of PPARγ2 and the pro-osteogenic capacity of IGF-I, we asked whether PPARγ2 was one of the determinants of *Igf1* expression in the skeletal micro-environment. We analyzed *Igf1* expression in a marrow stromal cell cell line, U-33 cells stably expressing PPARγ2 (U-33/γ2 cells) and found that PPARγ2 activation significantly suppressed *Igf1* mRNA transcripts and IGF-I protein levels in the conditioned media.[62] Similar results were also observed in bone marrow stromal cells treated with rosiglitazone. These lines of evidence demonstrated that the age-dependent increase of PPARγ2 expression in the skeletal micro-environment could play a role in skeletal loss through down-regulation of *Igf1* expression.

Nocturnin: A Downstream Regulator of PPARγ which Targets *Igf1*

Next, we tried to understand the possible mechanisms whereby PPARγ activation

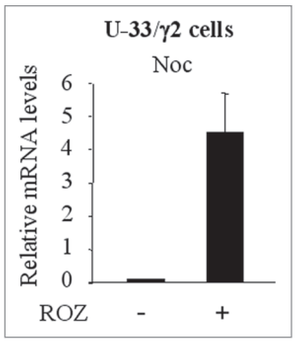

Figure 5. Upregulation of Noc in response to PPARγ2 activation. U-33 cells stably expressing PPARγ2 expression vector (U-33/γ2 cells) were treated with Rosiglitazone (ROZ) for 3 days and RNA was collected. Expression of Noc was analyzed by real-time PCR.

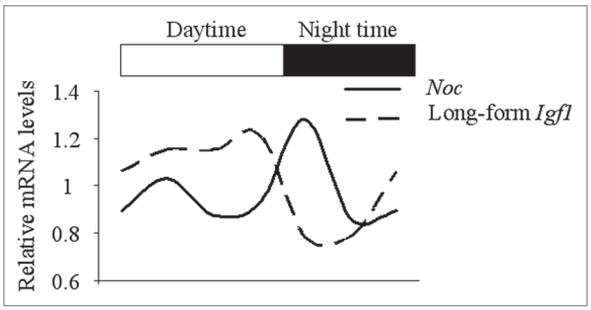

Figure 6. Anti-phase circadian expression pattern between Noc and Igf1 in the femur. RNA was collected from femur at the several time points of the day and expression of Noc and long-form Igf1 were analyzed by real-time PCR. White bar and black bar represent daytime (ZT 0–14) and night time (ZT 14–24), respectively.

suppressed *Igf1* expression. First, we examined the promoter region of the *Igf1* gene using data-based analysis to identify possible binding sites for PPARγ; however no candidate binding site was observed, suggesting that PPARγ regulation of *Igf1* was not at the transcriptional level. Based on these observations, we next performed micro-array analysis to detect genes affected by PPARγ2 in U-33/γ2 cells and found Noc to be one of the most highly upregulated genes with PPARγ2 activation by rosiglitazone (**Fig. 5**).[6,12]

As mentioned earlier, Noc functions as a deadenylase that degrades mRNA from poly-adenylation site of 3'UTR, thus adding a post-transcriptional regulation to gene expression. Importantly, RNA-binding proteins are required for Noc recruitment to RNA and function.[63] Igf1 transcripts contain a long 3' untranslated region (3'UTR).[64] The full length (6.4 kb) 3'UTR contains at least 3 poly-adenylation sites and the use of the first polyadenylation site generates a short-form (170-bp) 3'UTR.[64] Sequence analysis of the 3'UTR revealed that it is likely that the longer-form 3'UTR, not short-form 3'UTR, contains the regulatory regions where RNA-binding proteins interact. In addition, several lines of evidence suggest that *Igf1* transcripts with the longer-form of 3'UTR are more abundantly expressed than the short-form in skeletal tissue.[65,66] Based on these findings we hypothesized that Noc regulated Igf1 expression through recognition of the longer-form of 3'UTR in the skeleton.

To test this hypothesis, first, we analyzed the circadian expression profile of *Igf1* and *Noc* in whole femur.[67] *Igf1* showed circadian rhythmicity with the lowest expression at night when Noc transcripts were highest, demonstrating an anti-phase expression profile between these two genes (**Fig. 6**).[67] IGF-I protein levels in the bone marrow fluid was decreased at night time compared to the daytime.[67] Importantly, in Noc$^{-/-}$ femurs the reduction in Igf1 expression at night time was not observed, suggesting the important role of Noc in the circadian regulation of Igf1 in vivo. Second, to clarify whether the 3'UTR region of Igf1 mRNA is recognized by Noc, we generated a luciferase constructs containing the short form or full length of the 3'UTR region in the *Igf1* transcripts.[67] Noc overexpression did not have any effect on the 170 bp short 3'UTR, which included the first poly A site. However, luciferase activity of the long form 3'UTR (6 kb) was suppressed in Noc-overexpressing MC3T3-E1 cells, whereas it was markedly increased in MC3T3-E1 cells expressing shRNA for Noc. In sum Noc recognizes the long form of the *Igf1* 3'UTR and apparently suppresses *Igf1* transcripts by deadenylation, possibly in collaboration with one or more RNA binding proteins. This novel post-transcriptional mechanism for regulating IGF-I may be operative during various environmental stresses including caloric restriction and post-operative catabolic conditions.

Finally, we sought to determine whether the age-associated increase in PPARγ that is responsible for the enhanced recruitment of adipocytes into the bone marrow

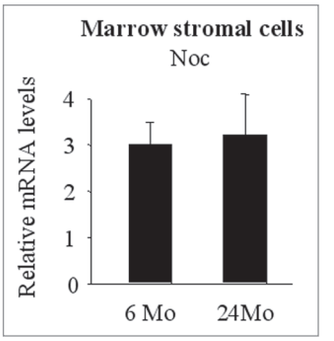

Figure 7. Noc expression was unchanged during the process of aging in the bone marrow stromal cells. Bone marrow stromal cells were collected from adult (6 months old) and old (24 months old) mice and expanded. Noc expression was measured by real-time PCR.

was related to Noc-induced deadenylation of IGF-I in the skeleton. Despite earlier studies showing that Noc was one of the most upregulated genes in the aging rodent liver,[68] we were unable to demonstrate any effect of aging on Noc expression in bone marrow stromal cells or whole bone (**Fig. 7**). This is not inconsistent with our earlier findings that Noc may be upstream rather than directly downstream of PPARγ, but it also suggests there are other factors produced locally that affect mesenchymal cell fate. Given the fact that Noc is highly upregulated by PPARγ activation (such as rosiglitazone, high-fat diet[69]), it is possible that Noc could be more operative in pathogenic conditions such as obesity, type 2 diabetes and corticosteroid use all of which affect circadian rhythms. In addition, the persistent expression of Noc early in adipogenic differentiation, even in cells from old mice, provides support

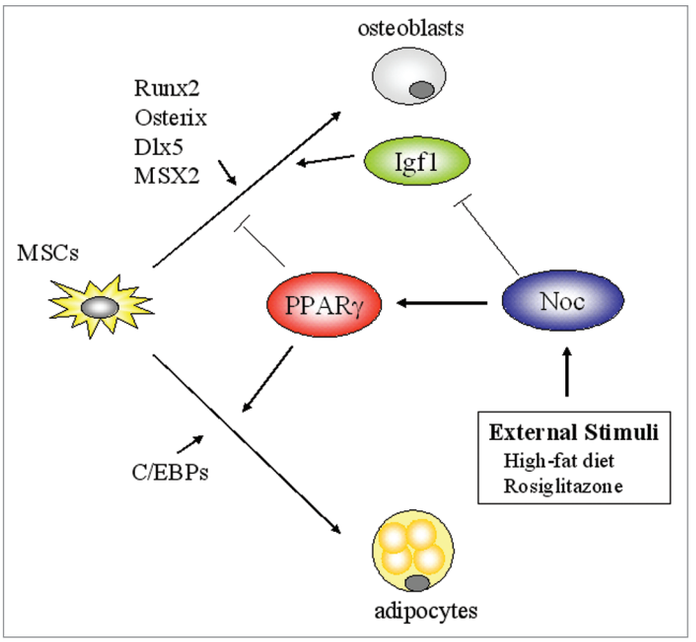

Figure 8. Schematic model of Noc, PPARγ and Igf1 network in the bone marrow. The fate of mesenchymal stem cells (MSCs) is regulated by a number of transcription factors. PPARγ and C/EBPs regulates the specification of MSCs toward the adipogenic lineage, while Runx2, Osterix and Dlx5 favors osteoblastogenesis. Circadian-regulated gene, Nocturnin (Noc), increases PPARγ activity in part by stimulating its nuclear translocation and enhances adipogenesis. In contrast, Noc is a negative regulator for osteoblastogenesis. Noc downregulates Igf1 expression probably through targeting the long-form 3' UTR of Igf1 transcripts, resulting in the decrease in IGF-I protein levels in the skeletal microenvironment. Because IGF-I is a pivotal factor for skeletal metabolism, Noc-induced bone loss may be in part explained by its activity to decrease IGF-I levels. Importantly, Noc is induced by external stimuli such as high-fat diet and rosiglitazone, thus proposing the possibility that Noc is a circadian factor linking external stimuli and cellular metabolic outputs. PPARγ, Peroxisome proliferator-activated receptor-gamma; C/EBP, CCAAT enhancer binding protein; Runx2, Runt-related transcription factor 2; Dlx5, Distal-less homebox homolog 5; Msx2, Muscle segment homeobox homolog of 2.

for the proposition that circadian regulation at the cellular level is still active during advanced aging. On the other hand, it is also possible that the absence of an increase in Noc with aging in the marrow favors a different adipocyte program with functional relevance; i.e., promotion of fatty acid storage rather than utilization of these compounds. Several lines of evidence from our studies of the Noc molecule and the Noc$^{-/-}$ mice suggest that it is important in brown adipogenesis. If PPARγ increases but Noc does not with aging, it is conceivable that the marrow adipocytes observed in old mice are functionally different from other fat cells. Two critical pieces of evidence are lacking to date: (1) other RNA targets for Noc as a deadenylase and (2) how Noc determines the functional adipocyte program when it promotes PPARγ activation.

Conclusion

PPARγ activity in response to TZDs is different than PPARγ activity during aging. Although both activities lead to increased adipogenesis there are major functional differences. TZD-induced activity has beneficial effects on energy metabolism, while aging- or fatty acids-induced PPARγ activity have a net negative effect leading to decrease in insulin sensitivity and increased inflammatory markers. Understanding the role of Nocturnin in the aging process is critical, particularly since this protein may differentially impact brown adipogenesis more than white. Future studies are designed to address these considerations and should inform us further about aging and the adipocyte program.

Acknowledgements

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